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SOLID DISPERSION OF RESVERATROL SUPPORTED ON MAGNESIUM DIHYDROXIDE (RESV@MDH) MICROPARTICLES IMPROVES ORAL BIOAVAILABILITY

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Abstract. Resveratrol, because of its low solubility in water and its high membrane permeability, is collocated in the second class of the biopharmaceutical classification system, with limited bioavailability due to its dissolution rate. Solid dispersion of resveratrol supported on magnesium dihydroxide (RESV@MDH) was evaluated to improve solubility and increase bioavailability of resveratrol. Fluorimetric microscopy and granulometric analysis display three types of microparticles with similar size: type 1 that emitted preferably fluorescence at 463 nm (λ_{ecc} 358 nm), type 2 that emitted preferably fluorescence at 605 nm (λ_{ecc} 550 nm) andtype 3 that are non-fluorescent. Micronized pure resveratrol display only microparticles type 1 whereas type 3 are associated to pure magnesium dihydroxide. Dissolution test in simulated gastric environment resveratrol derived from RESV@MDH in comparison to resveratrol alone displayed better solubility. According to the biopharmaceutical classification, an increase of 3 fold of resveratrol bioavailabilitywas observed after oral administration of 50 mg/Kg of resveratrol of RESV@MDH in rabbits. We hypothesize that type 2 microparticles represent magnesium dihydroxide microparticles with a resveratrol shell and that they are responsible for the improved resveratrol solubility and bioavailability of RESV@MDH.

Keywords: resveratrol, magnesium dihydroxide, solubility, bioavailability, dissolution rate, microparticles

INTRODUCTION

Resveratrol (trans-3,5,4'-tri-hydroxic-stilbene) is a stilbenic structure polyphenol, initially isolated from the route of the white hellebore (VeratumGrandiflorumO.Loes) and later from the root of the Polygonumcuspidatum, a plant used in traditional Chinese and Japanese medicine. Resveratrol becomes popular in 1992 when it was suggested that it could be the reason behind red wine's cardio-protective effects (french paradox; [1]), and its popularity increases in 1997 when it was proved that resveratrol is able to prevent colon-rectal cancer in mice [1]. Resveratrol based compounds present anti-oxidant, anti-inflammatory, anti-viral, cardio-protective, neuro-protective, anti-cancer and anti-angiogenetic activities [1-3]. It has been recently observed in healthy obese human subjects that treatment with trans-resveratrol reduces glucose, tryglicerides and inflammation markers levels with a similar effect to the one induced by caloric restriction [4]. Action mechanism of resveratrol has not been completely defined yet, and for this reason recently studies have been carried out in order to understand the aspects that are still not clear. The induction of the caloric restriction, which is thought be at the base of resveratrol's multiple effects,has been associated to the increase of cyclic adenosine monophosphate's levels (cAMP) resulting from the inhibition of type IV phosphodiesterase (PDE4)caused by resveratrol. The increase of cAMP activates protein kinase A (PKA) and the proteic exchanger EPAC which together activate protein kinase activated by AMP (AMPK). The activity of AMPK together with that of PKA increases the functionality and the expression of Sirtuin1 (SIRT1) which regulates the mitochondrial energetic production and the re-order of lipidic metabolism [5].

Resveratrol is poorly bioavailable because of reduced absorption mainly due to its low solubility and fast metabolism that converts its into glucuronide and sulfates compounds [1,6]. In humans resveratrol can be detected in plasma about 30 minutes after oral administration, meaning that its absorption already starts at the gastric level and reaches a plasmatic submicromolar concentration peak. Such peak is variable and hardly related to the used dose. For example, by administrating a 25 mg dose of resveratrol a 10 ng/ml plasmatic concentration is obtained, while increasing such dose by 20 times (500 mg/die) its plasma level increases only 7 times (72.6 ng/ml) [7]. Differences in resveratrol absorption have been demonstrated by clinical trials based on the oral administration of 150 mg/die of resveratrol for a prolonged period of time. It has been observed that the same dose produces different plasmatic concentrations: 231 ng/ml [4] and 24.8ng/ml [8]. Several strategies have been performed to increase its bioavailability and to allow itspotential in health properties. A recent revision of the literature highlights how the increasedbioavailability of resveratrol is a necessary element in order to evaluate the real pharmaceutical and health potential of this well-known polyphenol [6]. According to the biopharmaceutical classification system (BCS) [9,10], resveratrol belongs to the second class which means that it is characterized by a low solubility in

water (about 30 mg/l), while it shares a high membrane permeability (log P~3.1) [11].Among the different strategies, new formulations have been developed that are able to increase its apparent solubility for example by using a lipophilic vehicle [12] or through various processes such as the complexation with cyclodextrins [13], nanopreparation, or mycellarsolubilization with biliary acid [11]. It has been demonstrated, *in vitro* studies, that the increase of apparent resveratrol solubility allows a partial saturation of the mechanisms that are involved in its metabolism (conjugation) with a subsequent increase of resveratrol's bioavailability [14]. This is in accord to BCS for molecules class II that increasing resveratrol apparent solubility produces a bioavailability improvement [9, 10, 15], but in a dedicate study the increase of solubility with cyclodextrinsdoes'tmodify bioavailability [13].

In the present study we investigated whether the solid dispersion of resveratrol on magnesium dihydroxide increasesits solubility and bioavailability indicating that in some instance this approach could be exploited to enhance biological properties of resveratrol. Although resveratrol does not display chelating properties, some studies have shown its ability to interact with heavy metals such as copper, zinc and aluminum [16, 17].In this present work we report further evidences of the interaction between metal and resveratrol, and the chance to use this to increase resveratrolbioavailability.Specifically we demonstrated that resveratrol interacts with magnesium dihydroxide at the microparticle level and that this is able to modify its bioavailability.

MATERIAL AND METHODS

Solid dispersion of resveratrol on magnesium dihydroxide preparation.

Magnesium dihydroxide and resveratrol (from *Polygomuncuspidatum,* 98% pure)solids dispersion was performed bymodified co-precipitation method of Biswicka [18]. Briefly, nucleation and growth of magnesium dihydroxide and resveratrol particles was obtained by mixing a hydro alcoholic solution (water-ethanol 1:1) of magnesium chloride and resveratrol (mass ratio of magnesium dihydroxide:resveratrol 70:30)with sodium hydroxide to keep thepH of solution between 10-11 during the co-precipitation.The solid precipitate wasseparated through centrifugation, washed with deionized water/ethanol, dried out at 50 °Cand thenmicronized.Magnesium dihydroxideon resveratrol solids dispersion described in this study was obtained by Good Manufacturing Practisechain by Prolabin&Tefarm, Ponte Felcino (PG) and distributed by S&R Farmaceutici SpA, Bastia Umbra (PG), with trade name, Revifast®. The resveratrol content in the solid dispersion was evaluated by HPLC methods (see below). The mean value obtainedin the three samples was about 30% of total weight.

Particle size analysis.

The size of the particles has been carried out by a Malvern Mastersizer 2000, a laser diffraction particle size analyzer, on the dried powders.

Supersaturation assays

A weighted amount of RSV@MDH and of Resveratrol were placed in series of closed flat-bottomed glass vessels containing 250 ml of simulated gastric fluid (pH 1.2, USP XX). The vessels were inserted in shaking water bath (Nuve ST 30) at 37 °C and 110 rpm for 2 hrs. At appropriate times (1, 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min) 2 ml samples were withdrawn and replaced by fresh dissolution medium, then filtered (Spartan 13/02 RC, Whatman GmbH, Dassel, Germany) and analyzed. The drug concentration was determined by HPLC (see below).

Field emission Scanning Electron Microscopy

The morphology of the samples was investigated with a FEG LEO 1525 scanning electron microscope (FE-SEM). FE-SEM micrographs were collected by depositing the samples on a stub holder and after a sputter coating with chromium for 20 seconds.

Microscopic fluorescence analysis of powders was performed using an AxioEsaminer(Zeiss) fluorescence microscope with a digital camera Axio Cam 502 Mono. Dry powders were observed both directly and as fine dispersions obtained by mixing powder with glycerol. Samples have been exposed with DAPI filter for 200 ms, and withRhodamine filter for 3000 ms.Imagines acquisition and analysis has been led with Zen 2 software (Zeiss).

***In vivo* absorption test.**The trial was carried out at the experimental farm of the University of Batumi, Georgia. Rabbits were exposed to a continuous photoperiod of 16 h light per day at 40 lx. Room temperature ranged from 18 to 27◦C. Fresh water was always available. Animals were fed with 130 g/day of a standard diet. The experimental protocol was approved by the Local Ethical Committee for Animal Experimentation at the University Batumi, Georgia. All efforts were made to minimize animal distress and to use only the number of animals necessary to produce reliable results. The tests were conducted on New Zealand White hybrid rabbits (4.5-5 kg weight range). Two groups of 4 animals each were prepared for the comparative treatment of resveratrol (pure resveratrol versus RESV@MDH). The rabbits were fasted for 24 h before oral administration of a suspension containing 50 mg/kg of pureresveratrol or 50 mg/kg of resveratrol from RESV@MDH. The powders were suspended in 10 ml of a glucose solution and oral administered to a conscious animal by a syringe

(time zero). At 0,5, 15, 30, 90, 120 and 180 minutes, blood samples were taken (about 2 ml) through the auricular artery and put in heparinized tubes. The samples were centrifuged at 2500 g for 5 minutes and the plasma was recovered. Acetonitrile was added to the plasma samples (v:v 1: 1 ratio) and left for 5 minutes in order to precipitate plasma proteins. After centrifugation the supernatant was recovered for the dosage of resveratrol by HPLC.

HPLC analysis.

The measurements were performed by an Agilent HPLC 1200 series equipped with an Agilent Zorbax SB C18 4.6x250mm 5-µm Agilent P / N 880975-902 column. Elution was carried out under isocratic conditions using as mobile phase (Water + 0.1% v / v Trifluoroacetic acid) / (Acetonitrile + 0.1% v / v Trifluoroacetic acid) = 65/35, with a flow of 1ml / min and a column temperature of 30 ° C. 20 µL of samples were injected, after 0.2micron Nylon membrane filtration, and the analytes were detected by VWD Detector, λ = 306 nm. For the quantification of resveratrol a calibration was performed that allowed to detect the polyphenol at a retention time of 5.6 min with a detection limit of 4 ng/ml. All the plasma concentrations were multiplied by 2 to take into account the dilution in acetonitrile during sample preparation and by 3.6 to take into account the yield of extraction of resveratrol from plasma (28%) [19].

RESULTS

Microscopic analysis of Solid dispersion of resveratrol on magnesium dihydroxide

Powder was dispersed in glycerol and was observed at the microscope in brightfield illumination. The presence of particles with different scattering profile in a narrow range of size of few micrometrics was observed (Figure 1A). Fluorescence analysis of the samples with DAPI filter showed that about 10-20% of the microparticles emitted fluorescence light at about 463 nm when exited with light at λ_{ecc} 358 nm, these microparticleswere defined type 1 (Figure 1B). The mean size of type 1 is 1,8±0,1 µm, n=40 of diameter (since the not spherical morphology of the particles, the diameter it has been taken into account the longest diameter). When the sample was analyzed with Rhodamine filter (λ_e 605 nm , λ_{ecc} 550 nm), a comparable population of particles was visualized with a mean size of2,0±0,2 µm, n=34 and was named type 2 (Figure 1C). Type 1 microparticles display very scantly signals when observed with rhodamine filter and in same way the particles type 2 with DAPI filter. Finally, the majority of the microparticles doesn't display any fluorescence in every filters and these microparticles was define type 3 and they have medium size similar to other (Figure 1D).

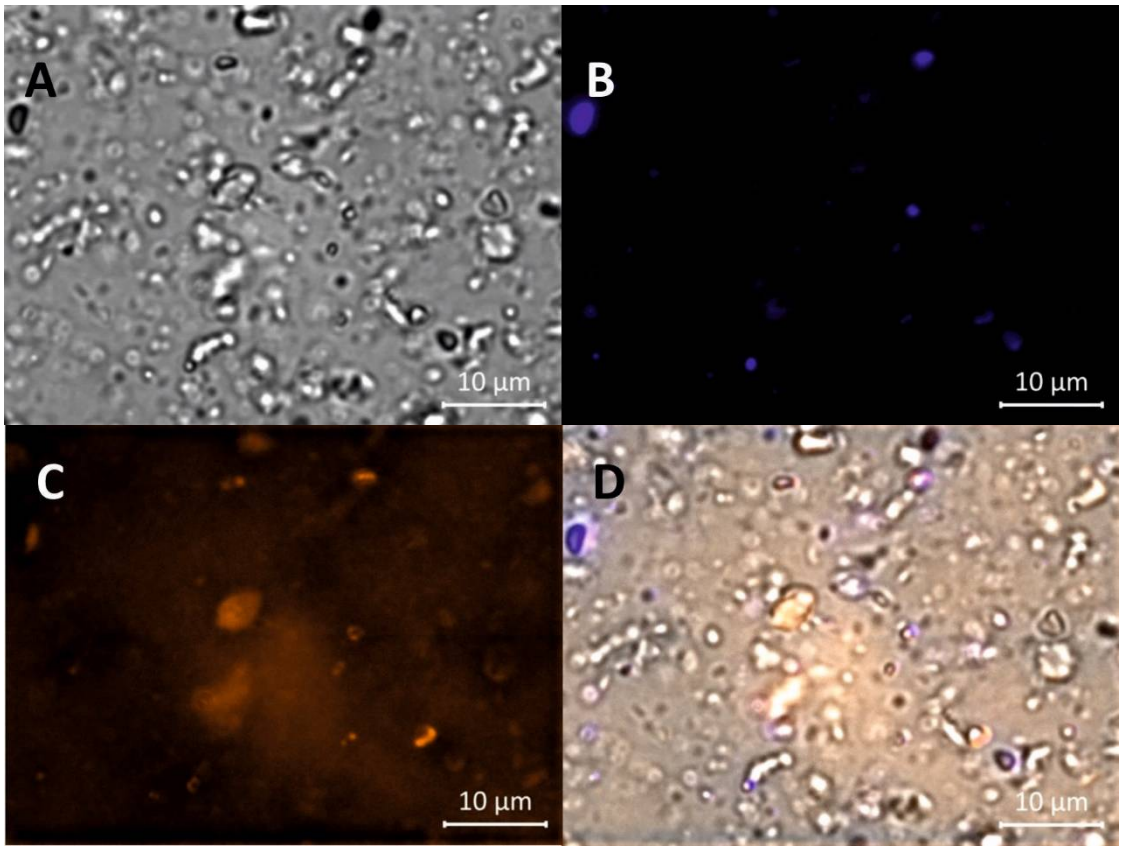

Figure 1. Image of RESV@MDH powder dispersed in glycerol under different eccitation sources. A: brightifield; B: DAPI fluorescence filter; C: Rohdamine fluorescence filter; D: merge of the previous three.

Thus, the solid dispersion of resveratrol on magnesium dihydroxideis composed by three distinct populations of microparticlesbased on the fluorescence profile. When we similarly analyzed the dry powder without dispersion in glycerolwe observed similar situation but aggregates of size around 5 µm are present as possible consequence of interaction ofthe three type of microparticles (Figure 2A). In accordance, the aggregated displays fluorescence signals from every channel.Granulometric and SEM analysis shows two distinct population size, one with size around 1 µm and the second population with size around 6 µm of diameter (Figure 2B and C).

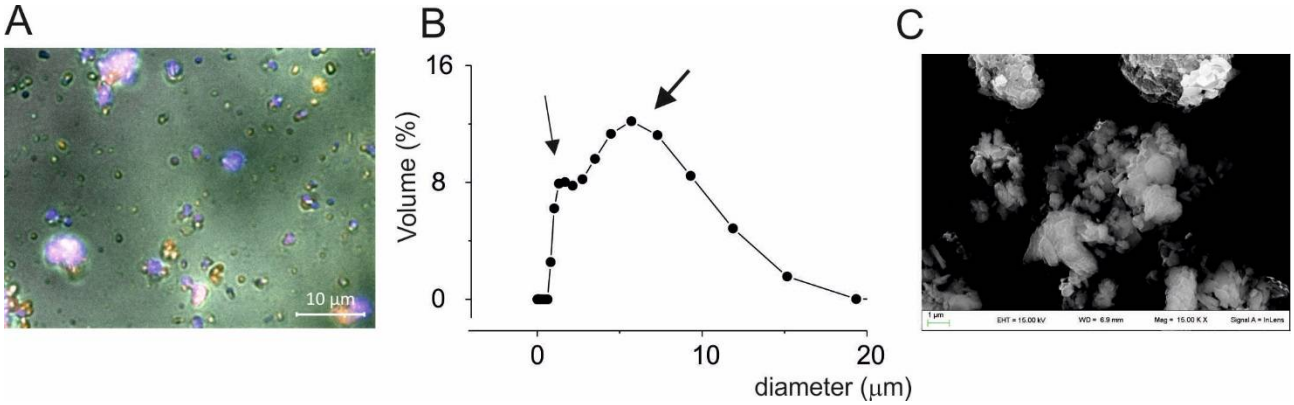

Figure 2. Properties of dry powder of RESV@MDH dry powder. A) Imagige resulted by digital merging of brightfield and DAPI/Rhodamine fluorescence illumination.B) granulometric analysis of RESV@MDH dry powder. C) SEM of RESV@MDH dry powder

Molecular nature of microparticles of Solid dispersion of resveratrol on magnesium dihydroxide. To define the molecular nature of different type of microparticles, we studied a powder of pure resveratrol micronizedwith similar distribution size of solid dispersion. Granulometricanalysisconfirmed thatresveratrolmicronized have the size of 1,6 µmof diameter (Figure 3A)similar to the particlessizemeasure inside the Solid dispersion of resveratrol on magnesium dihydroxide (see Figure 1 for comparison). Fluorescence microscopic analysis of the micronized resveratrol displays that all the microparticlesemittedfluorescence intensity as the particles type 1 (Figure 3B and C), whereas microparticles of type 2 ad 3 are not observed (Figure 3 D and E). Any fluorescence can be observed (DAPI and Rhodamine filters) during microscopic analysis of pure magnesium dihydroxide (brucite, data not shown). These datasuggest that the microparticles of type 1 are microparticles of pure resveratrol, whereas the microparticlestype 3 represent magnesium dihydroxide.

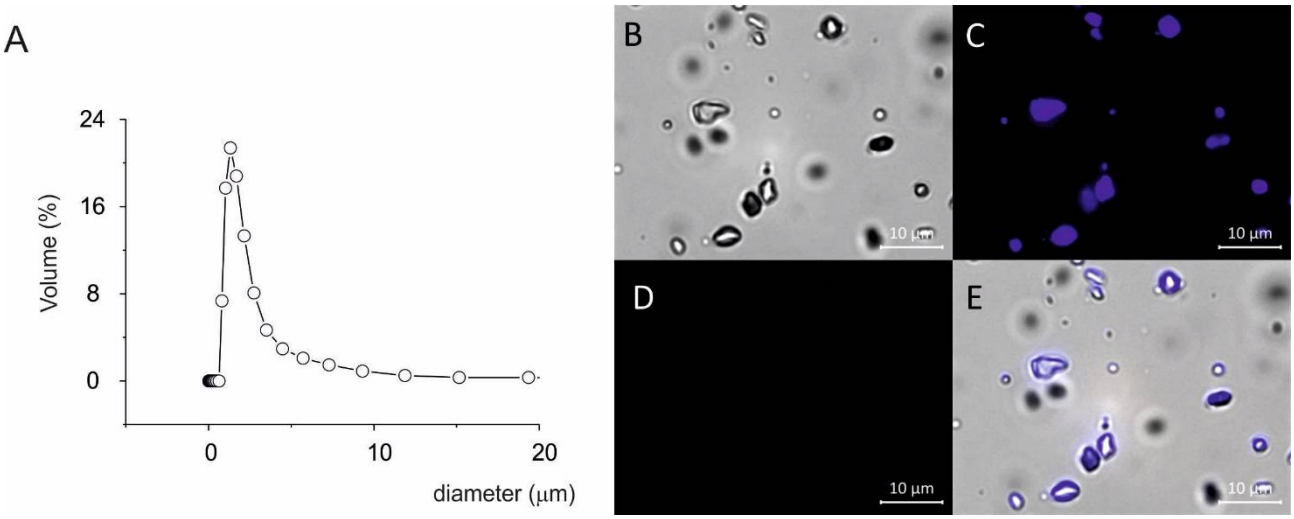

Figure 3. Properties of micronized resveratrol.A) granulometric analysis of microcrystalin resveratrol. B-E. Image of cristalline resveratrol powder dispersed in glycerol under different eccitation sources. B: brightifield; C: DAPI fluorescence filter; D: Rohdamine fluorescence filter; E: merge of the previous three.

Dissolution of Solid dispersion of resveratrol on magnesium dihydroxide. In Figure 4 dissolution profiles of RESV@MDH(red squares) and pure resveratrol (black squares) are presented (mg/L in function of time). The lines over-exposed represent the best fit of experimental data with exponential equation $C(t)=Cmax * (1 – exp(-t/\tau))$, Cmax = maximum solubility value; t = time; τ = time in which dissolution reaches about 63% of maximum process.The equation represents a form studying the dissolution profiles according to Weibull's models (21). The best data fit are for Cmax: 40.8 and 13 mg/L for RESV@MDHand resveratrol respectively while τ was0.4 and 2.2 minutes for RESV@MDHand resveratrol respectively This data indicate that RESV@MDH shows a dissolution rate five times higher than resveratrol (compare τ) and a maximum solubility three times as big (compare Cmax). To verify if magnesium chloride participates to major solubility (Cmax)of resveratrol by forming a complex, we verified the interaction between them by performingspectrophotometric profile of resveratrol alone or in presence of magnesium ion in acid environment. It is possible to see in figure 4B, that the addition of magnesium doesn't significantly modify the UV absorption spectra, suggesting that the magnesium doesn't interact with resveratrol and that the major solubility was dependent on other factors.

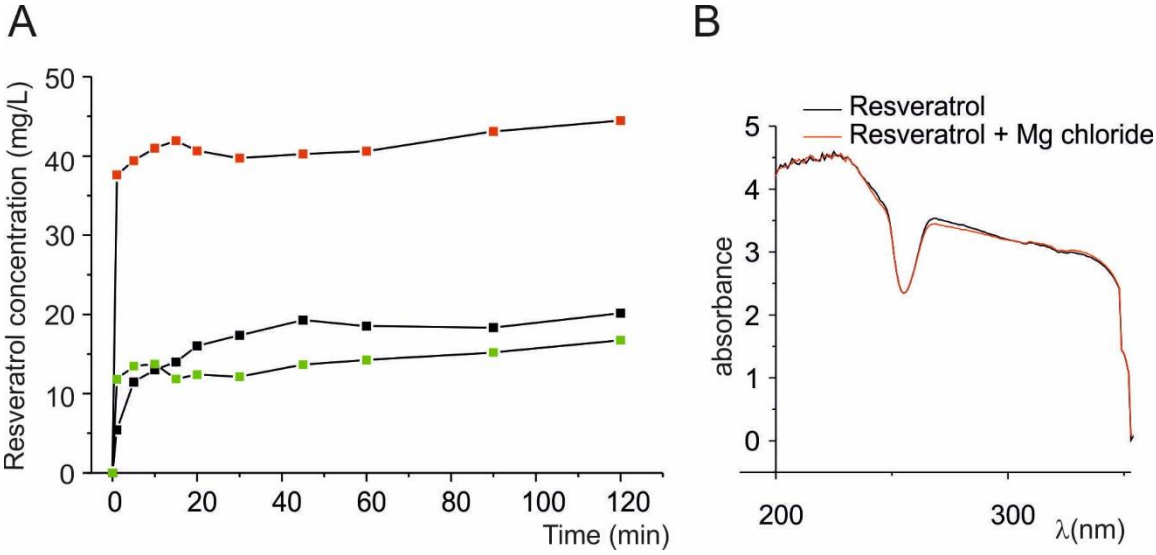

Figure 4. Solubility of resvferatrol from RESV@MDH and it interaction with magnesium ion.A dissolution test of pure resveratrol powder versus solid dispersion on magnesium dihydroxide. B) UV/Vis Absorbance spectroscopy for the study of Resveratrol. Black: 0.008 mM Resveratrol in ethanol:water (75:25, v/v) in 100 mMHCl;Red: 0.008 mM Resveratrol in ethanol:water (75:25, v/v) + 100 mMHCl + 0.008 mM of MgCl.

Pharmacokinetic profile of solid dispersion of resveratrol on magnesium dihydroxide. The mean plasma concentration of resveratrol following oral administration of 50 mg/kg of RESV@MDH and pure resveratrol was investigated in the rabbit animal model. Resveratrol plasma concentration versus time curves from administration is display in Figure 5. Pharmacokinetic variables derived from this pharmacokinetic profile are summarized in table 1. Resveratrol is virtually absent in animal plasma prior to oral administration (time 0) but it seemed to be rapidly absorbed with a peak of maximal concentrations ($\mathbf{C}_{max}$) at between 15 and 30 min post-dose. The $\mathbf{C}_{max}$ of resveratrol was 76.3 ng/ml and 101.3 ng/ml for resveratrol and RESV@MDH respectively. At 30 to 90 minutes from the administration, the resveratrol plasma concentration of RESV@MDH treated animal results statistically greater (p about 0.1) compared to resveratrol treated animal and at 180 minutes the resveratrol is no longer detectable in the plasma of both groups of animals. The values of Area Under Curve (AUC) of the plasma concentration profile until time 3h was2698 ng and 8944 ng for resveratrol and RESV@MDH respectively. These data demonstrate an enhancement of bioaviability of 3.3 fold (ratio of $AUC_{RESV@MDH}$/ $AUC_{resveratrol}$, Table 1).

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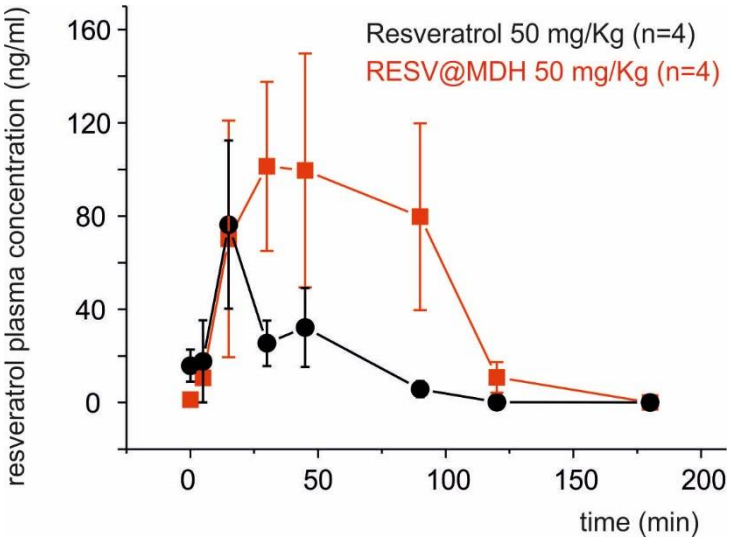

Figure 5. Pharmacokinetic profiles of resveratrol after oral administrationin rabbits. Groups of 4 animals each were treated with resveratrol (50 mg/Kg of pure resveratrol versus RESV@MDH). Blood samples taken at 0.5, 15, 30, 90, 120 and 180 minutes.

Table1: Pharmacokineticparameters of oral administration of 50 mg/Kg of resveratrol from pure resveratrol and from **RESV@MDH**.

Parameters	**Resveratrolo 50 mg/Kg**	**RESV@MDH (resveratrol 50 mg/Kg)**	**Increase%**
AUC (Area Under Curve)	2698 ng/ml*min	8944 ng/ml*min	330
Time to plasmaticpeak	15 minuts	30 minuts	200
Peakduration	25 minuts	105 minuts	420
Cmax	76.3 ng/ml	101.3 ng/ml	130

DISCUSSION

Solid dispersion of resveratrol on magnesium dihydroxide (RESV@MDH) represents a new formulation that possess an increased solubility of resveratrol (spring form). RESV@MDHis able to solubilize itself faster and in greater amounts with respect to resveratrol, with remarkable advantages in biopharmaceutical terms and therefore of bioavailability. From the physical point of view it is a poly disperse granular material, where the active is supported by inorganic material with a high safety level (magnesium hydroxide). Furthermore, this improves its performances without modifying

chemically the natural product's structure. RESV@MDH^{TM}allows to obtain an apparent solubility much higher with respect to resveratrol as a consequence of an increased dissolution rate and of the establishment of over-saturation phenomena due to different energetic states of resveratrol (Figure 6).This dispersion is formed by three types of microparticles that we define as type 1, 2 and 3. Based to results we hypnotized that microparticles type 1 and 3 represent resveratrol and magnesium dihydroxide crystals, respectively. The unexpected result is the presence of microparticles type 2 that probably represent the form responsible of enhanced properties of the solid dispersion. Based to the evidence of change of its fluorescence, we suggest that a fraction of resveratrol forms a shell around magnesium dihydroxidemicroparticles. The better solubility of resveratrol displayed by solid dispersioncould beexplained by coexistence of two energetic states of resveratrol related two type of microparticles observed (type 1 and 2, Figure 6). It is possible to exclude the involvement of free magnesium ions (Mg++) in better solubility of resveratrol since their absorbance spectrum of was not modify by presence of metals in acid environment (Figure 4 B). The state of over-saturation could lead to the major absorption (increase of the gradient concentration) and therefore toa major bioavailability [20].

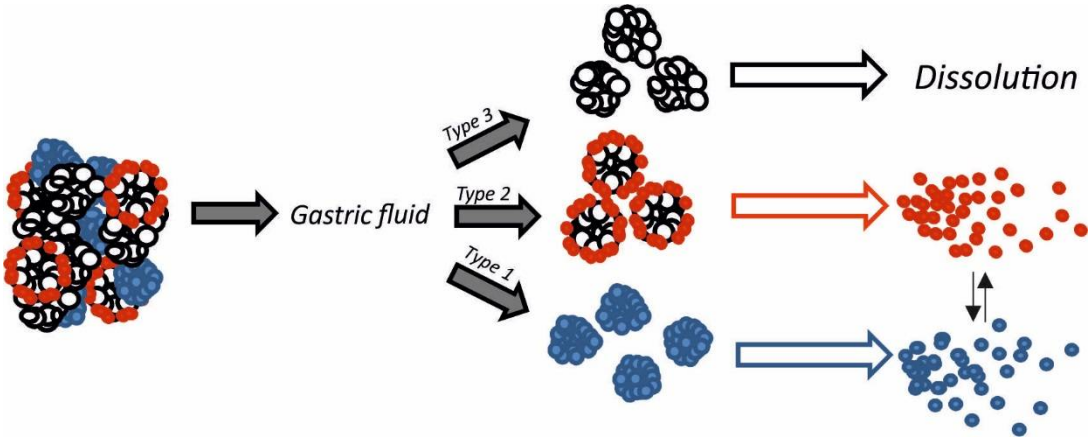

Figure 6. Scheme hypothetical dissolution events that occur to RESV@MDH powder when it is in contact with simulate stomach fluids. Big powder aggregates divide into main microparticles named as type 1, 2 and 3. In acid milieu the type 3 microparticles of magnesium dihydroxide completely dissolve; type 1 microparticles (blue) dissolve following the normal dissolution rate in water solution together with type 2 microparticles (red). In this case the limiting step in resveratrol release could be related to acid erosion of dihydroxide core.

The reduced and homogeneous particle size represent parameters that improve the dissolution rate observed for RESV@MDHis according to Noyes and Whitney law [21].The comparative dissolution rate of resveratrol displayed in figure 5 demonstrates that the sovrasaturation state is not dependent

from particles size resveratrol. Further study are need to clarify the mechanisms of better solubility of solid dispersion of resveratrol on magnesiumdihydroxide. The RESV@MDH represent a new way to uncover the therapeutic potential of resveratrol with possible application as anti-inflammatory, anti-viral, cardio-protective, neuro-protective, anti-cancer and anti-angiogenetic agents [1-3]. As regard the anticancer properties, resveratrol was demonstrated to increase the effect of radio and chemotherapeutic agents [22] in particular against glioblastoma cancer cells [23].

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Conflicts of Interest

RS and BF are co-inventors of the patent EPO n EP20130425091; RGI is an employee of S&R Farmaceutici, whom hold the rights and licence of REVIFAST®. All other authors declare no conflict of interest.

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